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(54) Melanoma associated antigenic polypeptide, epitopes thereof and vaccines against melanoma

Melanoma-assoziierte Antigene, Epitope davon und Impstoffe gegen Melanoma

Antigène associé au mélanome, épitopes de cet antigène et vaccins contre le mélanome

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Description

[0001] The present invention is concerned with cancer treatment and diagnosis, especially with a melanoma associated antigen, epitopes thereof, vaccines against melanoma, tumour infiltrating T lymphocytes recognizing the antigen and diagnostics for the detection of melanoma and for the monitoring of vaccination.

[0002] Tumor cells may emancipate themselves from restrictive growth control by oncogene activation, and/or by the inactivation of tumor suppression genes. The course of tumor progression proceeds by a series of gradual, stepwise changes in different 'unit characteristics', i.e. phenotypic traits, many of which are known to be determined or at least influenced by the altered expression of defined oncogenes and/or tumor suppressive genes. Emancipation of the cell from immunological host restriction may follow multistep pathways similar to the emancipation from growth control.

[0003] A problem often encountered in cancer immunotherapy is the lack of immunogenicity of the tumor. This escape of the immune control system can be understood on basis of phenotype differences encountered in neoplastic cells (differences found in Burkitt's lymphoma cells according to Klein, G. and Boon, T., Curr. Opinion in Immunol. 5, 687-692, 1993):

- decreased ability to process and present antigens;
- decreased ability to stimulate autologous T cells;
- complete downregulation of immunogenic proteins associated with transformed cells;
- no or low expression of leukocyte adhesion molecules or other accessory molecules; and
- selective downregulation of certain MHC class I and class II alleles.

[0004] MHC Class I/II antigens are often downregulated in solid tumors. This may affect all class I/II antigens, or only part of them. Viral and cellular peptides that can sensitize appropriate target cells for cytotoxic T lymphocyte mediated lysis may fail to do so when produced in cells with a low level of expression of MHC class I antigen. Cytotoxic sensitivity may be induced, at least in some cases by raising the level of MHC class I/II antigen expression by interferon γ and tumor necrosis factor α.

[0005] However, during the stepwise changes from normal to tumor tissue tumor-associated antigens appear. These antigens can be exposed through various mechanisms:

- they can be molecules that are masked in some way during normal cell development, but where the neoplastic change induces removal of the masking protection for the immunosystem;
- deletion of some molecules from the plasma membrane may alter the profile of adjacent molecules in a given membrane patch, and thus, in effect generate a new profile that might become immunogenic to the host;
- a membrane alteration accompanying neoplastic transformation may expose new, previously hidden regions of a molecule, or may result in addition of new structural features to an existing molecule.
- shedding and disintegration of tumor cells may expose the immune system to nuclear, nucleolar, or cytoplasmic components that are normally hidden in the cell.

[0006] The characteristics of tumor-associated antigens are very much dependent on the origin of the tumor carrying them. The existence of antigens associated with animal tumors was documented in the last century, and the antigenic character of human cancers has been well established, primarily through recent studies with monoclonal antibodies.

[0007] Attempts to isolate and chemically characterize these antigens have encountered serious difficulties, many having to do with a lack of reagents suitable for precipitation of the antigen-bearing molecules from a solution.

[0008] Like many other stimuli, the tumor-associated antigens activate not one but a whole set of defense mechanisms - both specific and unspecific, humoral and cellular. The dominant role in in vivo resistance to tumor growth is played by T lymphocytes. These cells recognize tumor-associated antigens presented to them by antigen presenting cells (APC's), and will be activated by this recognition, and upon activation and differentiation, attack and kill the tumor cells. A special class of these sort of lymphocytes is formed by the tumor infiltrating lymphocytes (TIL's) which can be found in solid tumors.

[0009] It has already been suggested (EP 147,689) to activate T lymphocytes with an antigenic substance linked to an insoluble carrier in vitro and then to administer these activated lymphocytes to a tumor patient.

[0010] Conventional chemotherapy is relatively ineffective in the treatment of patients with metastasic melanoma, and approximately 6000 patients die of this disease in the United States each year.

[0011] Rosenberg et al. (New Eng. J. Med. 319(25), 1676-1681, 1988) have shown the beneficial effect of immunotherapy with autologous TIL's and interleukin-2 (IL-2) in melanoma patients.

[0012] This therapy constitutes of resection of the tumor deposit, isolation of the TIL's, in vitro expansion of the TIL's and infusion into the patient under concurrent treatment of high and toxicity inducing doses of IL-2.

[0013] The TIL's used by Rosenberg are directed to and able to recognize melanoma-associated antigens.

[0014] It has been our goal to isolate such a melanoma-associated antigen in order to be able to use the antigen and/or its epitopes for the development of an immunotherapy for melanoma patients.

[0015] Melanoma antigens have already been de-

scribed by Old, L. (1981) who identified 6 antigenic glycoproteins and 3 glycolipids occurring in 120 melanoma cell lines.

[0016] Also vaccines with melanoma antigens have been described: in US patent no. 5,030,621 and 5,194,384 a polyvalent vaccine has been made by culturing melanoma cells and subsequent isolation of excreted melanoma-specific antigens from the culture medium.

[0017] Some specific antigens have already been proposed for therapy and diagnosis of melanoma type of cancer: the peptide p97 has been disclosed in US 5,262,177 and US 5,141,742, while a 35 kD protein has been mentioned in EP 529,007.

[0018] We now have found a melanoma-associated polypeptide, characterized in that it comprises the aminoacid sequence of SEQ ID NO: 2.

[0019] This melanocyte lineage-specific antigenic polypeptide (also mentioned gp100) is recognized by the monoclonal antibody NKI-beteb, which antibody has proven suitable for diagnostic purposes. The antigens recognized by this antibody are intracellular proteins of approximately 10 kd (gp 10) and 100 kd (gp100). The latter is also detectable in a culture medium of melanoma cells (Vennegoor, C. et al, Am. J. Pathol. 130, 179-192, 1988). It has also been found that the gp100 antigen reacts with other melanoma-specific antibodies such as HMB-50 (described by Vogel, A.M. and Esclamado, R.M., Cancer Res. 48, 1286-1294, 1988) or HMB-45 (described by Gown, A.M. et al., Am. J. Pathol. 123, 195-203, 1986). Since the proteins reacting with these monoclonal antibodies have been shown te be glycosylated in melanoma cells, differences have been found in mobility when analyzed by SDS-PAGE.

[0020] Although this gp100 antigen is predominantly expressed intracellularly, it has now been established that it is a suitable immunogenic antigen, because it has been demonstrated that these intracellular proteins can be processed and presented as peptides in the context of MHC molecules to cells of the immune system. In fact, tumor infiltrating lymphocytes derived from tumors of melanoma patients have been found which react with the antigen.

[0021] Therefore, the gp100 polypeptide is a potential target for cellular responses against carcinoma and thus a suitable subject for therapy and diagnosis in melanoma patients.

[0022] Gp100 is a type I transmembrane protein, which has a threonine-rich domain containing repetitive amino acid sequences present in the middle of the protein (amino acids 309-427). This threonine-rich domain, which may be subjected to extensive O-linked glycosylation, is preceded by a histidine-rich region (amino acids 182-313) and followed by a cysteine-rich domain (amino acids 475-566). Based on hydrophobicity plot analysis (Kyte, J. and Doolittle, R.F., 1982), a single transmembrane domain bordered by charged residues is present in the carboxy-terminal part (amino acids 591-611) of gp100. The predicted cytoplasmic domain is 45 amino acids long. Five putative N-linked glycosylation sites are present, consistent with gp100 being a glycoprotein.

[0023] The term "polypeptide" refers to a molecular chain of amino acids, does not refer to a specific length of the product and if required can be modified in vivo or in vitro, for example by glycosylation, amidation, carboxylation or phosphorylation; thus inter alia peptides, oligopeptides and proteins are included within the definition of polypeptide.

[0024] Of course, functional derivatives as well as fragments of the polypeptide according to the invention are also included in the present invention. Functional derivatives are meant to include polypeptides which differ in one or more amino acids in the overall sequence, which have deletions, substitutions, inversions or additions. Amino acid substitutions which can be expected not to essentially alter biological and immunological activities, have been described. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M. D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous polypeptides.

[0025] Functional derivatives which still show immunological activity towards the monoclonal antibody NKI-beteb or HMB-50 or HMB-45 are included within the scope of this invention.

[0026] Furthermore as functional derivatives of these peptides are also meant peptides derived from gp100 which are able to induce target cell lysis by tumor infiltrating lymphocytes.

[0027] In addition, with functional derivatives of these peptides are also meant addition salts of the peptides, amides of the peptides and specifically the C-terminal amides, esters and specifically the C-terminal esters and N-acyl derivatives specifically N-terminal acyl derivatives and N-acetyl derivatives.

[0028] The polypeptides according to the invention can be produced either synthetically or by recombinant DNA technology. Methods for producing synthetic polypeptides are well known in the art.

[0029] The organic chemical methods for peptide synthesis are considered to include the coupling of the required amino acids by means of a condensation reaction, either in homogenous phase or with the aid of a so-called solid phase. The condensation reaction can be carried out as follows:

a) condensation of a compound (amino acid, peptide) with a free carboxyl group and protected other reactive groups with a compound (amino acid, pep-

tide) with a free amino group and protected other reactive groups, in the presence of a condensation agent;

b) condensation of a compound (amino acid, peptide) with an activated carboxyl group and free or protected other reaction groups with a compound (amino acid, peptide) with a free amino group and free or protected other reactive groups.

[0030] Activation of the carboxyl group can take place, inter alia, by converting the carboxyl group to an acid halide, azide, anhydride, imidazolide or an activated ester, such as the N-hydroxy-succinimide, N-hydroxy-benzotriazole or p-nitrophenyl ester.

[0031] The most common methods for the above condensation reactions are: the carbodiimide method, the azide method, the mixed anhydride method and the method using activated esters, such as described in The Peptides, Analysis, Synthesis, Biology Vol. 1-3 (Ed. Gross, E. and Meienhofer, J.) 1979, 1980, 1981 (Academic Press, Inc.).

[0032] Production of polypeptides by recombinant DNA techniques is a general method which is known, but which has a lot of possibilities all leading to somewhat different results. The polypeptide to be expressed is coded for by a DNA sequence or more accurately by a nucleic acid sequence.

[0033] It has been found that the amino acid sequence of gp100 closely resembles the amino acid sequence of the already known melanoma-associated peptide pMel17, disclosed in Kwon, B.S. (1991).

[0034] The amino acid differences between gp100 and Pmel17 consist of substitutions at amino acid position 274 (T-C/PRO-LEU) and 597 (C-G/ARG-PRO) and a stretch of 7 amino acid absent in gp100 at position 587. A single nucleotide difference at position 762 (C-T) does not result in an amino acid substitution. Gp100 is also 80% homologous to a putative protein deduced from a partial cDNA clone (RPE-1) isolated from a bovine retinal cDNA library (Kim, R.Y. and Wistow, G.J., 1992) and 42% homologous to a chicken melanosomal matrix protein, MMP115 (Mochii, M., 1991). See also fig. 2.

[0035] Also part of the invention is the nucleic acid sequence comprising the sequence encoding the gp100 polypeptide.

[0036] Preferably the sequence encoding gp100 is the sequence of SEQ ID NO:1.

[0037] As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in another codon still coding for the same amino acid, e.g. the codon for the amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of a polypeptide with an amino acid sequence shown in SEQ ID NO:2 use can be made of a derivate nucleic acid sequence with such an alternative codon composition thereby differring from the nucleic acid sequence shown in SEQ ID NO:1.

[0038] "Nucleotide sequence" as used herein refers to a polymeric form of nucleotides of any length, both to ribonucleic acid (RNA) sequences and to desoxyribonucleic acid (DNA) sequences. In principle this term refers to the primary structure of the molecule. Thus, this term includes double and single stranded DNA, as well as double and single stranded RNA, and modifications thereof.

[0039] The nucleotide sequence of gp100 contains 2115 base pairs (bp) and terminates with a poly(A) tract of 15 nucleotides which is preceded by the consensus polyadenylation sequence AATAAA. An open reading frame (ORF) extending from nucleotide 22 through 2007 is present in gp100 DNA. This ORF starts with an ATG codon within the appropriate sequence context for translation initiation and codes for a protein of 661 amino acids. The amino-terminal 20 amino acids fit all criteria for signal sequences, including a potential cleavage site after ALA at position 20 (-1), which would indicate that mature gp100 contains 641 amino acids (approximately 70 kD).

[0040] The most striking difference between gp100 and Pmel17 cDNAs is the inframe deletion of 21 bp in gp100 cDNA (fig. 2). Comparison of the nucleotide sequence of genomic DNA with the sequence of gp100 cDNA revealed the presence of an intron (102 bp) just at the position of the 21 bp insertion in Pmel17 cDNA. The exon/intron boundaries nicely fit the consensus 5' donor and 3' acceptor splice site sequences (Padgett, 1986). In the genomic DNA, the sequence comprising the additional 21 bp in Pmel17 cDNA is located directly upstream of the 3' cleavage site used to generate gp100 RNA and is preceded by an alternative 3' acceptor splice site. Whereas the gp100-specific 3' acceptor splice site fits the consensus sequence, the Pmell7-specific 3' acceptor splice site appears to be sub-optimal in that it lacks a pyrimidine-rich region. Sub-optimal RNA processing sites are present in many alternatively processed messenger RNA precursors and have been implicated to function in regulation of alternative RNA processing (reviewed by Green, M.R., 1991). Collectively, these data prove that the transcripts corresponding to gp100 and Pmel17 cDNAs are generated by alternative splicing of a single primary transcript.

[0041] A further part of the invention are peptides, which are immunogenic fragments of the gp100 polypeptide.

[0042] Immunogenic fragments are fragments of the gp100 molecule, which still have the ability to induce an immunogenic response, i.e. that it is either possible to evoke antibodies recognizing the fragments specifically, or that it is possible to find T lymphocytes which have been activated by the fragments.

[0043] As has been said above it has been known that the immunogenic action of tumor associated antigens is often elicited through a T cell activating mechanism (Townsend, A.R.M. and Bodmer, H., Ann. Rev. Immunol. 7, 601-624, 1989). Cytotoxic T lymphocytes (CTLs)

recognizing melanoma cells in a T cell receptor (TCR)-dependent and MHC-restricted manner have been isolated from tumor-bearing patients (reviewed by Knuth, A., 1992). Brichard et al. (1993) have shown that a peptide derived from tyrosinase, an other melanocyte-specific antigen, is recognized by a CTL clone.

[0044] It is known that the activation of T cells through the MHC molecule necessitates processing of the antigen of which short pieces (for example 8-12 mers) are presented to the T lymphocyte.

[0045] The immunogenic oligopeptides located in the gp100 sequence form also part of the invention.

[0046] We have found immunogenic peptide sequences of the gp100 sequence which are not only able to bind with the MHC I molecule, but which also have been demonstrated to recognize tumor infiltrating lymphocytes which have been isolated from a melanoma patient.

[0047] Several peptides have been found: the peptides having the amino acid sequences V-L-P-D-G-Q-V-I-W-V, M-L-G-T-H-T-M-E-V, R-L-M-K-Q-D-F-S-V, (V)-(W)-(K)-T-W-G-Q-Y-W-Q-V-(L) and L-L-D-G-T-A-T-L-R-L have been found to bind to the MHC HLA-A2.1 molecule. In addition, the latter two peptides are recognized by anti-melanoma cytotoxic T lymphocytes in the context of HLA-A2.1.

[0048] Preferably these peptides are flanked by non-related sequences, i.e. sequences with which they are not connected in nature, because it has been found that such flanking enhances the immunogenic properties of these peptides, probably through a better processing and presentation by APC's.

[0049] Another part of the invention is formed by nucleotide sequences comprising the nucleotide sequences coding for the above mentioned peptides.

[0050] Next to the use of these sequences for the production of the peptides with recombinant DNA techniques, which will be exemplified further, the sequence information disclosed in the sequence listings for gp100 or its epitopes can be used for diagnostic purposes.

[0051] From these sequences primers can be derived as basis for a diagnostic test to detect gp100 or gp100-like proteins by a nucleic acid amplification technique for instance the polymerase chain reaction (PCR) or the nucleic acid sequence based amplification (NASBA) as described in USP 4,683,202 and EP 329,822, respectively.

[0052] With PCR large amounts of DNA are generated by treating a target DNA sequence with oligonucleotide primers such that a primer extension product is synthesized which is separated from the template using heat denaturation and in turn serves as a template, resulting in amplification of the target sequence. When RNA is to be amplified with PCR the RNA strand is first transcribed into a DNA strand with the aid of reverse transcriptase.

[0053] With the aid of NASBA large amounts of single stranded RNA are generated from either single stranded RNA or DNA or double stranded DNA. When RNA is to be amplified the ssRNA serves as a template for the synthesis of a first DNA strand by elongation of a first primer containing a ssRNA polymerase recognition site. The formed DNA strand in turn serves as the template for the synthesis of a second, complementary, DNA strand by elongation of a second primer, resulting in a double stranded active RNA-polymerase promoter site, and the second DNA serves as a template for synthesis of large amounts of the first template, the ssRNA, with the aid of RNA polymerase.

[0054] Detection of the amplified nucleotide sequence is established by hybridizing a complementary detection probe to the amplified nucleic acid. This probe can be labelled and/or immobilized on a solid phase. Detection of the label can be performed through methods known in the art. Detection of nucleic acids bound through the probe to the solid phase can be done by compounds capable of selective detection of nucleic acids.

[0055] As said before the nucleotide sequences can be used for the production of gp100 or one of its epitopes with recombinant DNA techniques. For this the nucleotide sequence must be comprised in a cloning vehicle which can be used to transform or transfect a suitable host cell.

[0056] A wide variety of host cell and cloning vehicle combinations may be usefully employed in cloning the nucleic acid sequence. For example, useful cloning vehicles may include chromosomal, non-chromosomal and synthetic DNA sequences such as various known bacterial plasmids, and wider host range plasmids such as pBR 322, the various pUC, pGEM and pBluescript plasmids, bacteriophages, e.g. lambda-gt-Wes, Charon 28 and the M13 derived phages and vectors derived from combinations of plasmids and phage or virus DNA, such as SV40, adenovirus or polyoma virus DNA (see also Rodriquez, R.L. and Denhardt (1988); Lenstra, 1990).

[0057] Useful hosts may include bacterial hosts, yeasts and other fungi, plant or animal hosts, such as Chinese Hamster Overy (CHO) cells or monkey cells and other hosts.

[0058] Vehicles for use in expression of the peptides will further comprise control sequences operably linked to the nucleic acid sequence coding for the peptide. Such control sequences generally comprise a promoter sequence and sequences which regulate and/or enhance expression levels. Furthermore an origin of replication and/or a dominant selection marker are often present in such vehicles. Of course control and other sequences can vary depending on the host cell selected.

[0059] Techniques for transforming or transfecting host cells are quite known in the art (see, for instance, Maniatis et al., 1982 and 1989).

[0060] It is extremely practical if, next to the information for the peptide, also the host cell is co-transformed

or co-transfected with a vector which carries the information for an MHC molecule to which said peptide is known to bind. Preferably the MHC molecule is HLA-A2.1, HLA-A1 or HLA-A3.1, or any other HLA allele which is known to be present in melanoma patients. HLA-A2.1 is especially preferred because it has been established (Anichini A., 1993) that melanoma cells carry antigens recognized by HLA-A2.1 restricted cytotoxic T cell clones from melanoma patients.

[0061] Host cells especially suited for the expression of gp100 are the murine EL4 and P8.15 cells. For expression of gp100 human BLM cells (described by Katano, M., 1984) are especially suited because they already are able to express the MHC molecule HLA-A2.1.

[0062] Gp100 or any of its peptides or their nucleotide sequences mentioned above can be used in a vaccine for the treatment of melanoma.

[0063] In addition to an immunogenically effective amount of the active peptide the vaccine may contain a pharmaceutically acceptable carrier or diluent.

[0064] The immunogenicity of the peptides of the invention, especially the oligopeptides, can be enhanced by cross-linking or by coupling to an immunogenic carrier molecule (i.e. a macromolecule having the property of independently eliciting an immunological response in a patient, to which the peptides of the invention can be covalently linked).

[0065] Covalent coupling to the carrier molecule can be carried out using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the peptides of the invention can be coupled, e.g. using water soluble carbodiimides such as dicyclohexylcarbodiimide, or glutaraldehyde.

[0066] Coupling agents such as these can also be used to cross-link the peptides to themselves without the use of a separate carrier molecule. Such cross-linking into polypeptides or peptide aggregates can also increase immunogenicity.

[0067] Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

[0068] Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F(R) or Marcol 52(R)), saponins or vitamin-E solubilisate.

[0069] The vaccine according to the present invention can be given inter alia intravenously, intraperitoneally, intranasally, intradermally, subcutaneously or intramuscularly.

[0070] The useful effective amount to be administered will vary depending on the age and weight of the patient and mode of administration of the vaccine.

[0071] The vaccine can be employed to specifically obtain a T cell response, but it is also possible that a B cell response is elicited after vaccination. If so, the B cell response leads to the formation of antibodies against the peptide of the vaccine, which antibodies will be directed to the source of the antigen production, i.e. the tumor cells. This is an advantageous feature, because in this way the tumor cells are combatted by responses of both immunological systems.

[0072] Both immunological systems will even be more effectively triggered when the vaccine comprises the peptides as presented in an MHC molecule by an antigen presenting cell (APC). Antigen presentation can be achieved by using monocytes, macrophages, interdigitating cells, Langerhans cells and especially dendritic cells, loaded with one of the peptides of the invention. Loading of the APC's can be accomplished by bringing the peptides of the invention into or in the neighbourhood of the APC, but it is more preferable to let the APC process the complete gp100 antigen. In this way a presentation is achieved which mimicks the in vivo situation the most realistic. Furthermore the MHC used by the cell is of the type which is suited to present the epitope.

[0073] An overall advantage of using APC's for the presentation of the epitopes is the choice of APC cell that is used in this respect. It is known from different types of APC's that there are stimulating APC's and inhibiting APC's.

[0074] Preferred are the listed cell types, which are so-called 'professional' antigen presenting cells, characterized in that they have co-stimulating molecules, which have an important function in the process of antigen presentation. Such co-stimulating molecules are, for example, B7, CTLA-4, CD70 or heat stable antigen (Schwartz, 1992).

[0075] Fibroblasts, which have also been shown to be able to act as an antigen presenting cell, lack these co-stimulating molecules.

[0076] It is also possible to use cells already transfected with a cloning vehicle harbouring the information for gp100 and which are cotransfected with a cloning vehicle which comprises the nucleotide sequence for an MHC class I molecule, for instance the sequence coding for HLA A2.1, HLA A1 or HLA A3.1. These cells will act as an antigen presenting cell and will present gp100-fragments in the MHC class I molecules which are expressed on their surface. It is envisaged that this presentation will be enhanced, when the cell is also capable of expressing one of the above-mentioned co-stimulating molecules, or a molecule with a similar function. This expression can be the result of transformation or transfection of the cell with a third cloning vehicle having the sequence information coding for such a co-stimulating molecule, but it can also be that the cell already was capable of production of co-stimulating molecules.

[0077] In stead of a vaccine with these cells, which next to the desired expression products, also harbour many elements which are also expressed and which can

negatively affect the desired immunogenic reaction of the cell, it is also possible that a vaccine is composed with liposomes which expose MHC molecules loaded with peptides, and which, for instance, are filled with lymphokines. Such liposomes will trigger a immunologic T cell reaction.

[0078] By presenting the peptide in the same way as it is also presented in vivo an enhanced T cell response will be evoked. Furthermore, by the natural adjuvant working of the, relatively large, antigen presenting cells also a B cell response is triggered. This B cell response will amongst others lead to the formation of antibodies directed to the peptide-MHC complex. This complex is especially found in tumor cells, where it has been shown that in the patient epitopes of gp100 are presented naturally, which are thus able to elicit a T cell response. It is this naturally occurring phenomenon which is enlarged by the vaccination of APC's already presenting the peptides of the invention. By enlarging not only an enlarged T cell response will be evoked, but also a B cell response which leads to antibodies directed to the MHC-peptide complex will be initiated.

[0079] The vaccines according to the invention can be enriched by numerous compounds which have an enhancing effect on the initiation and the maintenance of both the T cell and the B cell response after vaccination.

[0080] In this way addition of cytokines to the vaccine will enhance the T cell response. Suitable cytokines are for instance interleukines, such as IL-2, IL-4, IL-7, or IL-12, GM-CSF, RANTES, tumor necrosis factor and interferons, such as IFN-.

[0081] In a similar way antibodies against T cell surface antigens, such as CD2, CD3, CD27 and CD28 will enhance the immunogenic reaction.

[0082] Also the addition of helper epitopes to stimulate $CD4^+$ helper cells or $CD8^+$ killer cells augments the immunogenic reaction. Alternatively also helper epitopes from other antigens can be used, for instance from heat shock derived proteins or cholera toxin.

[0083] Another part of the invention is formed by usage of gp100 reactive tumor infiltrating lymphocytes (TIL's). In this method the first step is taking a sample from a patient. This is usually done by resection of a tumor deposit under local anaesthesia. The TIL's present in this specimen are then expanded in culture for four to eight weeks, according to known methods (Topalian, S.L. et al., 1987). During this culture the TIL's are then checked for reactivity with gp100 or one of the epitopes derived from gp100. The TIL's which recognize the antigen are isolated and cultured further.

[0084] The tumor infiltrating lymphocytes, reactive with gp100, which are obtained through this method, form also part of the invention. One such TIL cell line, designated TIL 1200, has been found which specifically reacts with gp100 and its epitopes. This TIL 1200 cell line also expresses the MHC molecule HLA-A2.1. Furthermore expression of TCR α/β, CD3 and CD8 by this cell line has been demonstrated. Furthermore TIL 1200 recognizes transfectants expressing both HLA-A2.1 and gp100.

[0085] This TIL 1200 and other TIL's recognizing gp100 are suited for treatment of melanoma patients. For such treatment TIL's are cultured as stated above, and they are given back to the patients by an intravenous infusion. The success of treatment can be enhanced by pre-treatment of the tumor bearing host with either total body radiation or treatment with cyclophosphamide and by the simultaneous administration of interleukin-2 (Rosenberg, S.A. et al., 1986).

[0086] The TIL's infused back to the patient are preferably autologous TIL's (i.e. derived from the patient's own tumor) but also infusion with allogenic TIL's can be imagined.

[0087] A further use of the TIL's obtained by the method as described above is for in vivo diagnosis. Labelling of the TIL's, for instance with ^{111}In (Fisher, 1989) or any other suitable diagnostic marker, renders them suited for identification of tumor deposits in melanoma patients.

[0088] Another part of the invention is formed by the T cell receptor (TCR) expressed by gp100 reactive CTLs. As is well known in the art, the TCR determines the specificity of a CTL. Therefore, the cDNA encoding the TCR, especially its variable region, can be isolated and introduced into T cells, hereby transferring anti-tumor activity to any T cell. Especially introduction of such a TCR into autologous T cells and subsequent expansion of these T cells, will result in large numbers of CTL suitable for adoptive transfer into the autologous patient.

[0089] Also cells harbouring this T cell receptor can be used for vaccination purposes.

[0090] A vaccine can also be composed from melanoma cells capable of expression of gp100. It is possible to isolate these cells from a patient, using anti-gp100 antibodies, such as NKI-beteb, but is also possible to produce such melanoma cells from cultured melanoma cell lines, which either are natural gp100-producers or have been manipulated genetically to produce gp100. These cells can be irradiated to be non-tumorogenic and infused (back) into the patient. To enhance the immunologic effect of these melanoma cells it is preferred to alter them genetically to produce a lymphokine, preferably interleukine-2 (IL-2) or granulocyte-macrophage colony stimulation factor (GM-CSF). $Gp100^+$ melanoma cells can be transfected with a cloning vehicle having the sequence coding for the production of IL-2 or GM-CSF.

[0091] Infusion of such a vaccine into a patient will stimulate the formation of CTL's.

[0092] Another type of vaccination having a similar effect is the vaccination with pure DNA, for instance the DNA of a vector or a vector virus having the DNA sequence encoding the gp100 antigen or peptides derived therefrom. Once injected the virus will infect or the DNA will be transformed to cells which express the antigen or the peptide(s).

[0093] Antibodies to any gp100 peptide, including an-

tibodies to (V)-(W)-(K)-T-W-G-Q-Y-W-Q-V-(L), and L-L-D-G-T-A-T-L-R-L are also part of the invention.

[0094] Monospecific antibodies to these peptides can be obtained by affinity purification from polyspecific antisera by a modification of the method of Hall, R. et al. (1984). Polyspecific antisera can be obtained by immunizing rabbits according to standard immunisation schemes.

[0095] Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogeneous binding characteristics for the relevant antigen. Homogeneous binding as used herein refers to the ability of the antibody species to bind to ligand binding domain of the invention.

[0096] The antibody is preferably a monoclonal antibody, more preferably a humanised monoclonal antibody.

[0097] Monoclonal antibodies can be prepared by immunizing inbred mice, preferably Balb/c with the appropriate protein by techniques known in the art (Köhler, G. and Milstein C., 1975). Hybridoma cells are subsequently selected by growth in hypoxanthine, thymidine and aminopterin in an appropriate cell culture medium such as Dulbecco's modified Eagle's medium (DMEM). Antibody producing hybridomas are cloned, preferably using the soft agar technique of MacPherson (1973). Discrete colonies are transferred into individual wells of culture plates for cultivation in an appropriate culture medium. Antibody producing cells are identified by screening with the appropriate immunogen. Immunogen positive hybridoma cells are maintained by techniques known in the art. Specific anti-monoclonal antibodies are produced by cultivating the hybridomas in vitro or preparing ascites fluid in mice following hybridoma injection by procedures known in the art.

[0098] It is preferred to use humanized antibodies. Methods for humanizing antibodies, such as CDR-grafting, are known (Jones, P.T. et al., 1986). Another possibility to avoid antigenic response to antibodies reactive with polypeptides according to the invention is the use of human antibodies or fragments or derivatives thereof.

[0099] Human antibodies can be produced by in vitro stimulation of isolated B-lymphocytes, or they can be isolated from (immortalized) B-lymphocytes which have been harvested from a human being immunized with at least one ligand binding domain according to the invention.

[0100] Antibodies as described above can be used for the passive vaccination of melanoma patients. A preferred type of antibodies for this kind of vaccine are antibodies directed against the above-mentioned peptides presented in connection with the MHC molecule. To produce these kind of antibodies immunization of peptides presented by APC's is required. Such an immunization can be performed as described above. Alternatively, human antibodies to peptide-MHC complexes can be isolated from patients treated with a vaccine consisting of APC's loaded with one of said peptides.

[0101] The antibodies, which are formed after treatment with one of the vaccines of the invention can also be used for the monitoring of said vaccination. For such a method serum of the patients is obtained and the antibodies directed to the peptide with which has been vaccinated are detected. Knowing the antibody titre from this detection it can be judged if there is need for a boost vaccination.

[0102] Specific detection of said antibodies in the serum can be achieved by labelled peptides. The label can be any diagnostic marker known in the field of in vitro diagnosis, but most preferred (and widely used) are enzymes, dyes, metals and radionuclides, such as ^{67}Ga, ^{99m}Tc, ^{111}In, ^{113m}In, ^{123}I, ^{125}I or ^{131}I,.

[0103] The radiodiagnostic markers can be coupled directly to the peptides of the invention or through chelating moieties which have been coupled to the peptide directly or through linker or spacer molecules. The technique of coupling of radionuclides to peptides or peptide-like structures is already known in the field of (tumor) diagnostics from the numerous applications of labelled antibodies used both in in vivo and in in vitro tests.

[0104] Direct labelling of peptides can for instance be performed as described in the one-vial method (Haisma, 1986). A general method for labelling of peptides through chelators, with or without linker or spacer molecules, has for instance been described in USP 4,472,509 and USP 4,485,086. Chelators using a bicyclic anhydride of DTPA have been disclosed in Hnatowich, D.J. et al. (1983). Coupling through diamide dimercaptide compounds has been disclosed in EP 188,256.

[0105] The present invention is further described by way of example with reference to the accompanying figures, in which:

Fig. 1 shows the genomic organization of part of the human gp100/Pmel17 gene. A and A' represent the introns which are removed in transcripts corresponding to gp100 cDNA and Pmel17 cDNA respectively. Exon sequences are indicated in capitals and intron sequences as small letters. The best fit to the branch point sequence (Ruskin, B. et al., 1984) is underlined.

Fig. 2 shows an alignment of the carboxyterminal part of members of the gp100/pMel17 family. identical amino acids (-) and gaps (*) are indicated. Conserved cysteine residues (#) are indicated as well.

Fig. 3. (A) Gp100 deletion mutants encoding parts of the gp100 protein are shown (numbers indicate amino acids in the gp100 protein as indicated in SEQ ID NO:2)

(B) Recognition by TIL 1200 of cells transfected with HLA-A2.1 and the gp100 deletion mutants shown in Figure 3A.

Fig. 4. (A) Five peptides derived from the gp100 148-166 region, varying from an 8-mer to an 11-mer, were tested for recognition by TIL 1200. Specific lysis was detected at an effector to target ratio of 30:1.

(B) Titration of gp100 peptides identified in Figure 4A for recognition by TIL 1200 (E/T ratio 30: 1).

Fig. 5. Binding of gp100 and viral epitopes to HLA-A2.1.

EXAMPLE 1 - MOLECULAR CHARACTERIZATION OF GP100

MATERIALS AND METHODS

Cells and monoclonal antibodies

[0106] The melanoma cell lines Mel-2a, M14, MEWO, BLM (Vennegoor et al., 1988; van Muijen et al., 1991; Bean et al., 1975; Katano et al., 1984) and the uveal melanoma cell line Mel 202 (Ksander et al., 1991) have been previously described. Isolation of normal human melanocytes from breast or foreskin was performed by the method of Eisinger and Marko (1982) with modifications by (Smit et al., 1993).

[0107] Mabs NKI-beteb and HMB-50 have been described previously (Vennegoor et al., 1988; Vogel and Esclamado, 1988). MAb HMB-45 was purchased from Enzo Biochem.

DNA constructs and transfections

[0108] The 2.2 kb Eco RI fragment containing gp100 cDNA was blunt-ended by filling in the ends with Klenow DNA Polymerase and then cloned in both orientations (pSVLgp100+ and pSVLgp100-) in the Sma I site of the eukaryotic expression vector pSVL (Pharmacia). pSVL contains the SV40 late promoter and polyadenylation site as well as the SV40 origin of replication, allowing a very high copy number during transient expression in COS-7 cells.

[0109] For the construction of the 3' truncated gp100 transcription unit pSVLgp100+(@BS) we deleted the sequence between the Bgl II site in the 3' part of gp100 cDNA and the Sac I site in the multiple cloning site of the vector. The resulting construct encodes a truncated gp100 protein in which the carboxy-terminal 133 amino acids of gp100 are replaced by 4 amino acids (Arg-Ile-Gln-Thr) encoded by vector sequences.

[0110] Transient expression of the constructs in COS-7 cells was performed by using 40 µg/ml lipofectin reagent from BRL (Felgner et al., 1987) and 7.5 µg DNA as described previously (Loenen et al., 1991).

Immunofluorescence

[0111] Transfected COS-7 cells were prepared for immunofluorescence 48 hours after the addition of the lipofectin/DNA mixture as described previously (Vennegoor et al., 1988). After incubation with the primary antibody for 45 minutes, cells were washed and incubated with fluorescein isothiocyanate (FITC)-labeled goat $F(ab)'_2$ anti-mouse IgG (Nordic) for 30 minutes. Preparations were examined using a confocal laser scanning microscope at 488 nm (Biorad MRC 600).

Metabolic labeling, Immunoprecipitations and V8 protease mapping.

[0112] Immunoprecipitation experiments were performed on metabolically labeled (L-[^{35}S]-methionine/cysteine; Amersham) cells as described by Vennegoor et al. (1988) using either mAb NKI-beteb or HMB-50 covalently linked to protein A-CL 4B sepharose beads (Pharmacia). In some experiments tunicamycin (75µg/ml , Calbiochem) was added during the pre-labeling period and remained present during the metabolic labeling reaction (12.5 minutes). Immunoprecipitates were analyzed under reducing conditions (5% β-mercaptoethanol in SDS-sample buffer) by SDS-PAGE using 5-17.5% polyacrylamide gradient gels. The relative molecular weight of the proteins was determined using co-electrophorised, pre-stained molecular weight markers (BRL). Gels were treated with 1 M sodium salicylate (pH 5.4) prior to autoradiography (Kodak XAR).

[0113] V8 protease mapping was performed using the digestion for proteins in gel slices procedure described by Cleveland et al. (1977). Briefly, gel slices containing the 100 kD proteins were placed in the wells of a second SDS-gel (10%) and overlayed with Staphylococcus aureus V8 protease (2,5 µg/sample, Miles laboratories). After electrophoresis gels were treated as described above.

Molecular cloning of part of the gp100/Pmel17 gene.

[0114] Part of the gp100/Pmel17 gene was amplified by PCR (Taq DNA Polymerase was from Gibco) on human genomic DNA isolated from peripheral blood lymphocytes (PBL's) using the following primers: 1497/1516: 5'-TATTGAAAGTGCCGAGATCC-3' and 1839/1857: 5'-TGCAAGGACCACAGCCATC-3' as described previously (Adema and Baas, 1991). The PCR products were subsequently amplified using a nested set of primers containing an additional Eco RI site (5'-TATCTAGAATTCTGCACCAGATACTGAAG-3' and 5'-TATCTAGAATTCTGCAAGATGCCCACGATCAG-3'). The underlined Eco RI sites in these primers were used to clone the PCR product in the Eco RI site of pUC 18.

RNA isolation and analysis

[0115] Total RNA was isolated using the guanidine thiocyanate procedure and centrifugation through a cushion of Cesium chloride (Chirgwin et al., 1979). cDNA was prepared using the Geneamp RNA PCR kit (Perkin Elmer Cetus) as indicated by the manufacturer. PCR analysis of the cDNAs was performed for 35 cycles in the presence of 3 mM $MgCl_2$ using primers 1497/1516 and 1839/1857 (see above) as described previously (Adema and Baas, 1991). The reaction products were size-fractionated on an agarose gel, blotted onto a nylon membrane (Hybond-N, Amersham) and hybridized to [^{32}P]-labeled oligonucleotide probes as described previously (Adema and Baas, 1991). As probes we used either a gp100-specific exon/exon junction oligonucleotide (5'-CTTCTTGACCAGGCATGATA-3') or a Pmel17- specific oligonucleotide (5'-TGTGAGAAGAATCCCAGGCA-3') which corresponds to 20 of the additional 21 nucleotides present in Pmel17 cDNA. In every hybridization experiment a spot blot containing an oligonucleotide comprising the Pmel17 exon/exon junction (5'-GCTTATCATGCCTGTGCCTGGATTCTTCTCACAGGT-3') was included as a control.

Nucleotide sequence analysis

[0116] Gp100 cDNA and genomic DNA clones were sequenced by the dideoxy-nucleotide sequencing method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia). The sequence of both strands was determined in each case. Since the genomic DNA clones were obtained after PCR, the sequence of four independent clones was determined. Analysis of the DNA sequence was performed using the University of Winconsin Genetics Computing Group sequence analysis programs (Devereux et al., 1984).

RESULTS

[0117] Expression of gp100 cDNA in non-pigmented COS-7 cells results in immunoreactivity with mAbs NKI-beteb, HMB-50 and HMB-45.

[0118] Expression of gp100 cDNA in gp100-negative BLM melanoma cells results in immunoreactivity with the melanocyte lineage-specific mAbs, NKI-beteb, HMB-50 and HMB-45. To determine whether expression of gp100-c1 cDNA in non-melanocytic cells also results in immunoreactivity with these mAbs, we performed transient expression experiments in COS-7 cells (monkey kidney fibroblasts) with constructs containing gp100 cDNA in the coding or non-coding orientation. Only COS-7 cells transfected with the construct containing the cDNA in the coding orientation (COS-7/pSVLgp100+) react with all three mAbs. These data demonstrate that immunoreactivity with mAbs NKI-beteb, HMB-50 and HMB-45 after expression of gp100 cDNA is not restricted to melanocytic cells. In addition, these data show that the COS expression system can be used for further biochemical characterization of the proteins encoded by gp100 cDNA.

Analysis of the proteins encoded by gp100 cDNA.

[0119] To characterize the proteins encoded by gp100 cDNA, COS-7/pSVLgp100+ cells were metabolically labeled and subjected to immunoprecipitation with mAb NKI-beteb or HMB-50. MoAbs NKI-beteb and HMB-50 specifically immunoprecipitate proteins of approximately 100 kD (95-110 kd) from extracts of COS-7/pSVLgp100+ cells. The molecular weight of these proteins is similar (see also below) to those immunoprecipitated from extracts of metabolically labeled MEWO cells which express the antigens endogenously (Vennegoor et al., 1988). Consistent with previous reports (Vennegoor et al., 1988; Vogel and Esclamado, 1988), both mAbs also recognize a protein of 10 kD in extracts of MEWO melanoma cells. A protein of the same size reacts with mAb NKI-beteb in COS-7/pSVLgp100+ cells and can be discerned with mAb HMB-50 after prolonged exposure (not shown). We note that the amount of the 10 kd protein varied considerably between experiments. No specific proteins are immunoprecipitated by either of the mAbs from extracts prepared from COS-7 cells transfected with the construct containing the cDNA in the non-coding orientation.

[0120] Glycoproteins of approximately 100 kD reacting with mAbs NKI-beteb and HMB-50 have also been found in culture medium of melanoma cells (Vennegoor et al., 1988; Vogel and Esclamado, 1988). Comparison of the culture medium of metabolically labeled COS-7/pSVLgp100+ cells and MEWO cells reveals that both mAbs also recognize proteins of about 100 kD (see also below) in the culture medium of these cells. No proteins of 10 kD are immunoprecipitated by the mAbs from the culture medium of COS-7/pSVLgp100+ cells, as has been shown for melanoma cells. These data demonstrate that, as in melanoma cells, the proteins of about 100 kd recognized by mAbs NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells are secreted.

[0121] To exclude the possibility that the proteins detected by the mAbs are derived from endogenous genes induced after transfection with gp100 cDNA, we performed immunoprecipitation experiments with COS-7 cells expressing a 3' truncated gp100 transcription unit (see Materials & Methods for details). Proteins of approximately 85 kd are immunoprecipitated by both mAbs from COS-7 cells expressing this construct, consistent with a deletion of 129 amino acids. This finding provides direct evidence that the 100 kd protein recognized by mAbs NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells is encoded by gp100 cDNA.

The 100 kd protein encoded by gp100 cDNA is identical to gp100

[0122] The proteins of about 100 kD identified by mAbs NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells versus MEWO cells have a slightly different mobility when analyzed by SDS-PAGE. Since the proteins reacting with these mAbs have been shown to be glycosylated in melanoma cells (Vennegoor et al., 1988; Vogel and Esclamado, 1988), these differences could be due to altered glycosylation, an event frequently observed in the COS expression system. To confirm this, mAb NKI-beteb was used to immunoprecipitate proteins from MEWO cells and COS-7/pSVLgp100+ cells cultured in the presence of the glycosylation inhibitor tunicamycin. In both COS-7/pSVLgp100+ cells and MEWO cells the size of the proteins of about 100 kd is reduced to two protein bands of 90 kd and 85 kD, confirming that the observed difference in mobility is due to altered glycosylation.

[0123] To provide further evidence that the proteins recognized by mAb NKI-beteb in COS-7/pSVLgp100+ cells and MEWO cells are identical, we performed a V8 protease mapping experiment. The same protein fragments are obtained after V8 protease digestion of the major 100 kD protein isolated from COS-7/pSVLgp100+ cells or MEWO cells. We conclude from these data that gp100 cDNA encodes the melanocyte lineage-specific glycoprotein gp100 recognized by mAbs NKI-beteb and HMB-50 in melanoma cells.

Gp100 is a type I transmembrane protein highly homologous to Pmel17

[0124] The nucleotide sequence of gp100 cDNA was determined. It contains 2115 base pairs (bp) and terminates with a poly(A) tract of 15 nucleotides which is preceded by the consensus polyadenylation sequence AATAAA (Proudfoot and Brownlee, 1976). An open reading frame (ORF) extending from nucleotide 22 through 2007 is present in gp100 cDNA. This ORF starts with an ATG codon within the appropriate sequence context for translation initiation (Kozak, 1987) and codes for a protein of 661 amino acids (SEQ ID NO:1). The amino-terminal 20 amino acids fit all criteria for signal sequences, including a potential cleavage site after ALA at position 20 (von Heyne, 1986), which would indicate that mature gp100 contains 641 amino acids (approximately 70 kD). Based on hydrophobicity plot analysis (Kyte and Doolittle, 1982), a single transmembrane domain bordered by charged residues is present in the carboxy-terminal part (amino acids 591-611) of gp100. The predicted cytoplasmic domain is 45 amino acids long. Five putative N-linked glycosylation sites are present, consistent with gp100 being a glycoprotein. Furthermore, a histidine-rich domain (amino acids 182-313), a threonine-rich domain (amino acids 309-427) containing repetitive amino acid sequences, and a cysteine-rich domain (475-566 amino acids) are present.

[0125] A data base search (Pearson and Lipman, 1988; Altschul et al., 1990) revealed that gp100 is almost identical to Pmel17, another melanocyte-specific protein (Kwon et al., 1991). The amino acid differences between gp100 and Pmel17 consist of substitutions at position 274 (T-C/PRO-LEU) and 597 (C-G/ARG-PRO) and a stretch of 7 amino acid absent in gp100 at position 587 (see also figure 2). A single nucleotide difference at position 782 (C-T) does not result in an amino acid substitution. Gp100 is also 80% homologous to a putative protein deduced from a partial cDNA clone (RPE-1) isolated from a bovine retinal cDNA library (Kim and Wistow, 1992) and 42% homologous to a chicken melanosomal matrix protein, MMP115 (Mochii et al., 1991).

Gp100 and Pmel17 are encoded by a single gene

[0126] The most striking difference between gp100 and Pmel17 cDNAs is the inframe deletion of 21 bp in gp100 cDNA. One possible explanation for this difference is the existence of two closely related genes. However, since both cDNAs have identical nucleotide sequences in their 3' untranslated regions this explanation is not likely. Another possibility is that both cDNAs correspond to transcripts generated by alternative splicing of a single primary transcript. To test this hypothesis, we used PCR to analyze the genomic DNA corresponding to the part of the gp100 gene surrounding the putative alternative splice site. Comparison of the nucleotide sequence of this genomic DNA with the sequence of gp100-c1 cDNA revealed the presence of an intron (102 bp) just at the position of the 21 bp insertion in Pmel17 cDNA (Figure 1). The exon/intron boundaries nicely fit the consensus 5' donor and 3' acceptor splice site sequences (Padgett et al., 1986). In the genomic DNA, the sequence comprising the additional 21 bp in Pmel17 cDNA is located directly upstream of the 3' cleavage site used to generate gp100 RNA and is preceded by an alternative 3' acceptor splice site (Fig. 1). Whereas the gp100-specific 3' acceptor splice site fits the consensus sequence, the Pmel17-specific 3' acceptor splice site appears to be sub-optimal in that it lacks a pyrimidine-rich region (Fig. 1). Sub-optimal RNA processing sites are present in many alternatively processed messenger RNA precursors and have been implicated to function in regulation of alternative RNA processing (reviewed by Green, 1991). Collectively, these data prove that the transcripts corresponding to gp100 and Pmel17 cDNAs are generated by alternative splicing of a single primary transcript and thus originate from a single gene.

Expression of gp100 and Pmel17 RNAs in cells of the melanocytic lineage

[0127] The finding that gp100 and Pmel17 RNAs arise by alternative splicing of a single primary transcript, rais-

es the question whether this occurs in a developmentally regulated manner. An RNA species of 2.5 kb is the major RNA product detected by gp100 cDNA on Northern blots containing RNA isolated from melanocytic cells. The same results were obtained by Kwon et al. (1987) using Pmel17-1 cDNA as a probe. However, neither of the probes discriminate between gp100 and Pmel17 RNAs. To investigate the expression of gp100 and Pmel17 RNAs in cells of the melanocytic lineage, we performed a reverse transcriptase/polymerase chain reaction (RT/PCR) assay followed by Southern blotting and hybridization to either a gp100 specific exon/exon junction- or a Pmel17-specific oligonucleotide probe (see Materials & Methods). Gp100 and Pmel17 spliced products are both detected in 3 out of 4 cutanous melanoma cells, in uveal melanoma cells as well as in neonatal and adult melanocytes. No products are detected with either probe in gp100-negative BLM melanoma cells. These results demonstrate that in all melanocytic cells examined, gp100 and Pmel17 RNAs are expressed simultaneously.

EXAMPLE 2 - RECOGNITION OF GP100 BY TIL's

MATERIAL AND METHODS

Cell culture

[0128] TIL's were generated by growth of single cell suspensions of metastatic melanomas with 1,000 U/ml IL-2 (Cetus Corp., Emeryville, CA) and were grown as described previously (Kawakami, 1992). Melanoma cell lines Mel 397 and Mel 624 were obtained and grown as reported previously (Kawakami, 1992). HLA-A2.1$^+$ melanoma cell lines MeWo (Bean, 1975) and BLM (Katano, 1984) and murine P815 transfectants were grown in DMEM (Gibco, Paisley, Scotland, UK) plus 7.5% heatinactivated FCS (Gibco). JY, K562, and murine EL4 transfectants were cultured in Iscoves medium (Gibco) plus 7.5% FCS. Murine cells were grown in the presence of $5 \cdot 10^5$ M β-ME, and all media contained antibiotics. Isolation of normal melanocytes from foreskin was performed by the method of Eisinger and Marko (1982) with modifications as described previously (Smit, 1989). Melanocytes from passages two to three were used in chromium release assays.

DNA Constructs and transfection.

[0129] Plasmid pBJ1gp100neo was obtained by cloning the EcoRI fragment of a lambda gp100 cDNA clone in the coding orientation in the polylinker pBJ1-neo (Lin, 1990). Plasmid pBA2 containing a genomic fragment encoding HLA-A2.1 and human β-2 microglobulin was kindly provided by E.J. Baas (The Netherlands Cancer Institute, Division of Biochemistry, Amsterdam, The Netherlands). Plasmid pGK-hyg contains the hygromycin phosphotransferase gene (Te Riele, 1990). For the introduction of the HLA-A2.1 and human β-2 microglobulin genes, EL4 cells were transfected with 18 μg of pBA2 and 2 μg of pGK-hyg DNA according to the calcium phosphate coprecipitation procedure (Graham, 1973) using Calciumphosphate Transfection Systems (Gibco BRL, Gaithersburg, MD). 24 h after transfection, 500 μg/ml hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) was added to the medium for the selection of stable transfectants. HLA-A2.1$^+$ gp100$^+$ EL4 cells were obtained by transfection of stable HLA-A2.1$^+$ EL4 clones with 20 μg of pBJ1-gp100neo DNA by calcium phosphate coprecipitation and were selected with 1 mg/ml G418. P815 A2.1 and P815 A2.1/gp100 cells were kindly provided by P. Coulie (Ludwig Ins., Brussels, Belgium).

mAb and flow cytometry

[0130] Phenotypic analysis of melanomas, transfectants, and normal melanocytes was performed by indirect immunofluorescence followed by flow cytometry using a FACScan^R (Becton Dickinson & Co., Mountain View, CA). Purified anti-gp100 mAb NKI-beteb (Vennegoor, 1988) and anti-HLA-A2 mAbs BB7.2 (culture supernatant; Parham, 1981) and MA2.1 (ascites 1:500 dilution; Parham, 1978) were used as primary reagents. FITC conjugated GAM-IgG-F(ab')$_2$ (Zymed Laboratories, Inc. S. San Francisco, CA) was used for the second incubation. For the detection of the intracellular gp100 antigen cells were permeabilized in 0.01% digitonin and were subsequently fixed in 1% paraformaldehyde.

Chromium Release Assay

[0131] Chromium Release assays were performed as described previously (Kawakami, 1992). Briefly, 10^6 target cells were incubated with 100 μCi $Na^{51}CrO_4$ (Amersham Int., Bucks, UK) for 1 hour. Various amounts of effector cells were then added to $2 \cdot 10^3$ target cells in triplicate wells of U-bottomed microtiter plates (Costar, Badhoevedorp, The Netherlands) in a final volume of 150 μl. After 5 hours of incubation, part of the supernatant was harvested and its radioactive content measured. Target cells were incubated for 48 hours with 50 U/ml human (Boehringer, Ingelheim, Germany) or mouse recombinant IFN- (TNO, Rijswijk, The Netherlands) before use in chromium release assays.

TIL 1200

[0132] In search of gp-100 specific cytotoxic T lymphocytes (CTLs) we focused on HLA-A2.1 as a restriction element because of its widespread occurrence in caucasians and its presumptive dominant role in CTL reactivity against melanoma. A HLA-A2.1$^+$ TIL line, TIL 1200 (Shilyansky, J. et al, 1994), was used for this study. This TIL line expresses TCR α/β, CD3 and CD8.

RESULTS

HLA-A2.1-restricted killing of melanoma tumor cells by TIL 1200 corresponds to gp100 expression.

[0133] Cytolytic activity of TIL 1200 was analyzed using a panel of human melanoma cell lines. TIL 1200 efficiently lysed HLA-A2.1$^+$ Mel 624 and MeWo melanoma tumor cells, which both express gp100, whereas no reactivity towards HLA-A2.1$^-$ gp100$^+$ Mel 397 cells was seen. It is interesting to note that we observed that HLA-A2.1$^+$ BLM melanoma cells are also resistant to lysis by TIL 1200. Furthermore, HLA-A2.1$^+$ EBV-transformed B cells (JY), which also lack gp100 expression, and K562 cells, were not lysed by TIL 1200. Together, these data demonstrate that TIL 1200 displays HLA-A2.1-restricted killing which correlates with gp100 expression.

TIL 1200 recognizes HLA-A2.1$^+$gp100$^+$transfectants.

[0134] EL4 cells cotransfected with a genomic fragment encoding HLA-A2.1 together with a plasmid conferring hygromycine resistance were selected and analyzed by flow cytometry. HLA-A2.1 expressing cells were subsequently transfected with pBJ1-gp100neo, which encodes gp100 and confers resistance to G418. Stable transfectants were selected and were screened for gp100 expression using mAb NKI/beteb. In collaboration with P. Coulie a similar panel of transfectants was generated in murine P815 cells (P815 A2.1 and p815 A2.1/gp100). Using these murine transfectants as target cells in chromium release assay, we clearly observed gp100 specific lysis by TIL 1200. The percent specific lysis (25-35%, E/T 30:1) of murine EL4 A2.1/gp100 and P815 A2.1/gp100 transfectants by TIL 1200 was somewhat lower compared with that observed with HLA-A2.1$^+$ gp100$^+$ human melanoma cells (45-60%, E/T 30:1). This difference may be explained by non-matched accessory molecules between human TIL's and murine transfectants. To overcome this we introduced the gp100 antigen into human HLA-A2.1$^+$ gp100$^-$ BLM melanoma cells by transfection of pBJ1-gp100neo. Stable BLM gp100 clones were tested in chromium release assays using TIL 1200. BLM gp100 clones proved to be as sensitive to lysis by TIL 1200 as Mel 624 and MeWo cells which express the gp100 antigen endogenously. The gp100 specificity of TIL 1200 was further demonstrated by the absence of lysis of G418-resistant BLM cells not expressing gp100, excluding the possibility that neomycin-derived peptides are recognized.

EXAMPLE 3

Mapping of gp100 epitopes recognized by TIL 1200 using gp100 deletion mutants.

[0135] Basically, two methods are commonly used in the art to map epitopes recognized by anti-tumor CTL.

1. According to the HLA binding motifs peptides can be synthesized that reside in the target protein. These peptides can then be loaded onto cells bearing the appropriate restriction element, and used as targets for CTL.
2. Generation of deletion mutants and expression of these deletion mutants in for example COS-7 cells together with the appropriate restriction element. These transfected cells are then co-cultured with CTL and target cell lysis or TNF-α/IFNγ production by the CTL are measured. Transfectants not recognized by the CTL do not express the peptide.

[0136] Both methods have been done in search for the epitopes of the invention.

TIL 1200 mediated lysis of peptide loaded T2 cells.

[0137] We have chemically synthesized gp100 peptides potentially recognized by TIL 1200. Peptides were synthesized by a solid phase strategy on an automated multiple peptide synthesizer (Abimed AMS 422) using Fmoc-chemistry (Nijman, 1993). Actual binding of the peptides to HLA-A2.1 was established with a recently described peptide binding assay making use of processing defective T2 cells (Nijman, 1993). This analysis resulted in the identification of gp100 derived peptides that strongly bind to HLA-A2.1. Subsequently, T2 cells loaded with the peptides that strongly bind to HLA-A2.1 were subjected to lysis by TIL 1200 using a standard chromium release assay. In this way the peptide L-L-D-G-T-A-T-L-R-L has been identified according to this procedure.

EXAMPLE 4

gp100 Epitope identified by deletion mapping

[0138] Gp100 cDNA was inserted into expression vectors pBJ1neo, pCMVneo (Baker et al, 1990) and pSVL. For the generation of a gp100 cDNA lacking the coding sequences for the peptide 457-466, PCR reactions were performed with the following combinations of oligonucleotides:
5'-CATGGAAGTGACTGTCTACC-3' / 5'-CTGAGCGAATTCGGAACCTGTAATACTTTCCG-3', and 5'-CTGAGCGAATTCGTGAAGAGACAAGTCCCCC-3' / 5'-TCACAGCATCATATGAGAGTAC-3' using the full length gp100 cDNA as atemplate. PCR products were digested with Eco RI, ligated and served as a template for a nested PCR using the following primers: 5'-GCACAG-

GCCAACTGCAGA -3' / 5'-TTCAGTATCTGGTGCAGAAC-3'. The Kpn I-Cla I fragment from this PCR product was then exchanged with the corresponding fragment in pCMVgp100neo to generate pCMVgp100DEL454-481neo. Gp100 cDNA mutants DEL149-654 and DEL454-654 were obtained by deletion of the 1.7 kb Hind III and the 0.8 kb Eco RI fragments from pBJ1gp100DEL454-481neo, respectively. Gp100 cDNA mutants DEL100-654, DEL194-528 and DEL167-508 were obtained by deletion of the Bgl I-Sac I, Bamh HI-Bgl II and Apa I-Nsi I fragments from pSVLgp100 respectively.

[0139] BLM cells were transfected with 20 μg of pCMVgp100DEL454-481neo DNA according to the calcium phosphate coprecipitation procedure (Graham and van der Eb, 1973) using Calciumphosphate Transfection Systems (BRL, Gaithersburg, MD) and were selected with 1 mg/ml G418 (Gibco, Paisley, Scotland UK).

[0140] COS-7 cells were contransfected with 5 μg of pBJ1HLA-A2.1neo and 5 μg of pBJ1 or pSVL plasmids containing either full length or deleted gp100 cDNAs using the DEAE-dextran/chloroquine method (Seed and Aruffo 1987). After 48 hours of transfection COS-7 cells were used as stimulator cells in IFN-γ release experiments.

Release assays

[0141] Chromium release assays were performed as in Example 2.

[0142] For IFN- release assay 10^5 TIL 1200 responder cells were incubated together with $5\cdot10^4$ transiently transfected COS-7 stimulator cells in 300 μl medium in the presence of 100 U/ml IL-2 in a flat bottom 96 well microtiter plate. After 24 hours of incubation, 100 μl of supernatant was harvested and was screened for the presence of IFN-γ using a hIFN-γ-IRMA immunoradiometric assay kit (megenix Diagnostics SA, Fleurus, Belgium).

Results

[0143] Figure 3A shows the gp100 cDNA deletion mutants that were generated. As shown in Figure 3B, TIL 1200 specifically secreted IFN-γ when stimulated with COS-7 cells transfected with HLA-A2.1 and the full length gp100 cDNA. Again TIL 1200 reactivity was observed against the gp100DEL454-481 mutant. From the other gp100 deletion mutants, only the DEKL100-661 and DEL149-661 constructs were not recognized, thereby excluding the possibility that TIL 1200 was reactive with a peptide located N-terminal from amino acid position 148 in the gp100 protein. Also the C-terminal region of the gp100 protein protein could be excluded, because TIL 1200 reactivity could be observed using a mutant construct, DEL454-661, encoding the first 453 amino acids of gp100. From the observation that a construct coding within this N-terminal region upto amino acid 166 was able to stimulate TIL 1200 (DEL167-508), it was concluded that the epitope recognized was located between amino acids 148-166 of the gp100 protein.

HLA-A2.1 binding

[0144] Several motifs have been described for 9-mer or 10-mer peptides binding to HLA-A2.1 (Falk et al., 1991; Hunt et al., 1992; Ruppert et al., 1993) that were deduced from naturally processed and synthetic HLA-A2.1 binding peptides. The 148-166 region of the gp100 protein was screened against these motifs and a number of peptides were synthesized that fitted into a somewhat broader motif, including threonine residues at position two. These peptides were loaded onto HLA-A2.1$^+$ T2 cells and tested for their ability to induce TIL 1200 mediated target cell lysis (Figure 4A). The five tested peptides were all able to sensitize T2 cells for lysis by TIL 1200 when used at a concentration of 10 μg/ml. All these peptides contain the 8-mer peptide TWGQYWQV, corresponding to gp100 amino acids 155-162. All peptides were titrated to evaluate their relative ability to sensitize T2 target cells for lysis by TIL 1200. Figure 4B shows that the 9-mer peptide KTWGQYWQV can be recognized by TIL 1200 when applied at a concentration of 3 ng/ml, whereas the other peptides had to be applied at higher concentrations.

[0145] A comparison was made of the peptides KTWGQYWQV (gp100 amino acids 155-162), LLDGTATLRL (gp100 amino acids 457-466) and YLEPGPVTA (gp100 amino acids 280-288, identified by Cox et al., 1994) with three known viral epitopes presented in HLA-A2.1: the influenza matrix 58-66 peptide (Gotch et al., 1987), the HIV polymerase 510-518 peptide (Tsomides et al., 1991) and the HIV gp120 197-205 peptide (Dadaglio et al., 1991). The HLA-A2.1 binding capacity of the above mentioned epitopes was analyzed by means of an indirect binding assay using the processing defective cell line T2 (Nijman et al., 1993). Shortly: T2 cells were incubated with 12.5 μg of the epitopes. HLA-A2.1 stabilization at the cell surface was determined by flow cytometry using mAb BB7.2. The Fluorescence Index is expressed as the experimental mean fluorescence divided by the mean fluorescence that is obtained when T2 cells are incubated with a HLA-A2.1 non-binding peptide at a similar concentration.

[0146] Using this assay, a similar HLA-A2.1 stabilization with the gp100 280-288 epitope and the tested viral epitopes. Both epitopes of the invention (KTWGQYWQV and LLDGTATLRL) bind with a somewhat lower affinity to HLA-A2.1 (Figure 5). From this it is concluded that the gp100 epitopes bind to HLA-A2.1 with distinct affinities.

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Claims

1. Immunogenic peptide fragment of the melanoma

associated antigen of SEQ ID NO: 2, **characterized in that** said fragment comprises an amino acid sequence selected from the group consisting of L-L-D-G-T-A-T-L-R-L, V-L-P-D-G-Q-V-I-W-V, T-W-G-Q-Y-W-Q-V, V-W-K-T-W-G-Q-Y-W-Q-V, K-T-W-G-Q-Y-W-Q-V-L, K-T-W-G-Q-Y-W-Q-V and T-W-G-Q-Y-W-Q-V-L, wherein said immunogenic peptide is able to induce target cell lysis by tumor infiltrating lymphocytes.

2. Immunogenic peptide fragment according to claim 1, which comprises a deletion, a replacement, an inversion and/or an addition.

3. Immunogenic peptide fragment according to claim 2, wherein said replacement is selected from the group consisting of Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn and Ile/Val substitutions.

4. Immunogenic peptide fragment according to any one of claims 1-3, wherein said immunogenic peptide comprises a salt of the peptide, an amide of the peptide, an ester of the peptide, an N-acyl derivative of the peptide, or an N-acetyl derivative of the peptide.

5. Immunogenic peptide fragment according to claim 4, wherein said amide of the peptide comprises a C-terminal amide.

6. Immunogenic peptide fragment according to claim 4, wherein said ester of the peptide comprises a C-terminal ester.

7. Immunogenic peptide fragment according to claim 4, wherein said N-acyl derivative of the peptide comprises a N-terminal acyl derivative.

8. Nucleic acid sequence encoding an immunogenic peptide fragment according to any one of claims 1-3.

9. Nucleic acid sequence according to claim 8, **characterized in that** it comprises the nucleotide sequence selected from the group consisting of the sequences of SEQ ID NO: 3, 5, 7 and 9.

10. Cloning vehicle comprising the nucleic acid sequence of claim 8 or 9.

11. Host cell **characterized in that** it is transfected or transformed with the cloning vehicle according to claim 10, preferably cotransfected with a cloning vehicle comprising the nucleotide sequence coding for an MHC class I allele.

12. Host cell according to claim 11, **characterized in that** the host cell is selected from the group consisting of murine EL4 and P8.15 cells and human BLM cells.

13. Host cell according to claim 11 or 12, **characterized in that** it is an antigen presenting cell.

14. Host cell according to any one of claims 11-13, **characterized in that** it also produces co-stimulating molecules.

15. Vaccine **characterized in that** it comprises an immunogenic peptide according to any one of claims 1-7, or an epitope thereof.

16. Vaccine according to claim 15, **characterized in that** the peptide is mixed with a pharmaceutically acceptable carrier or diluents.

17. Vaccine according to claim 15 or 16, **characterized in that** it comprises an antigen presenting cell, which has been preloaded with the peptide.

18. Vaccine, **characterized in that** it comprises host cells according to any of claims 12-14.

19. Vaccine, **characterized in that** it comprises a nucleic acid according to claim 8 or 9.

20. Vaccine, **characterized in that** it comprises the T cell receptor against the peptides according to any one of claims 1-7, or cells expressing said T cell receptor.

21. Vaccine according to any one of claims 15-20, **characterized in that** it also comprises one or more compounds selected from the group consisting of an adjuvant, one or more cytokines, antibodies directed against CD2, CD3, CD27, CD28 or other T cell surface antigens and helper epitopes to stimulate CD4+ or CD8+ T cells.

22. Method for the generation of antigen reactive tumor infiltrating lymphocytes, **characterized in that** it comprises the steps of:

a. culturing tumor infiltrating lymphocytes present in a melanoma sample;
b. isolating the tumor infiltrating lymphocytes from the sample;
c. reacting said lymphocytes with an immunogenic peptide according to any one of claims 1-7; and
d. isolating the lymphocytes binding to said antigen.

23. Tumor infiltrating lymphocyte, **characterized in that** it is capable of binding to an immunogenic peptide according to any one of claims 1-7.

24. Vaccine, **characterized in that** it comprises tumor infiltrating lymphocytes according to claim 23.

25. Conjugate of a peptide and a detectable marker, **characterized in that** the immunogenic peptide according to any one of claims 1-7 is used.

26. Conjugate according to claim 25, **characterized in that** the detectable marker is a radionucleotide.

Patentansprüche

1. Immunogenes Peptidfragment des Melanom-assoziierten Antigens von SEQ ID Nr. 2, **dadurch gekennzeichnet, dass** das Fragment eine Aminosäuresequenz umfasst, die aus der Gruppe ausgewählt ist, die aus L-L-D-G-T-A-T-L-R-L, V-L-P-D-G-Q-V-I-W-V, T-W-G-Q-Y-W-Q-V, V-W-K-T-W-G-Q-Y-W-Q-V, K-T-W-G-Q-Y-W-Q-V-L, K-T-W-G-Q-Y-W-Q-V und T-W-G-Q-Y-W-Q-V-L besteht, wobei das immunogene Peptid die Lyse von Zielzellen durch tumorinfiltrierende Lymphocyten induzieren kann.

2. Immunogenes Peptidfragment gemäß Anspruch 1, das eine Deletion, eine Substitution, eine Inversion und/oder eine Addition umfasst.

3. Immunogenes Peptidfragment gemäß Anspruch 2, wobei die Substitution aus der Gruppe ausgewählt ist, die aus den Substitutionen Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn und Ile/Val besteht.

4. Immunogenes Peptidfragment gemäß einem der Ansprüche 1-3, wobei das immunogene Peptid ein Salz des Peptids, ein Amid des Peptids, einen Ester des Peptids, ein N-Acyl-Derivat des Peptids oder ein N-Acetyl-Derivat des Peptids umfasst.

5. Immunogenes Peptidfragment gemäß Anspruch 4, wobei das Amid des Peptids ein C-terminales Amid umfasst.

6. Immunogenes Peptidfragment gemäß Anspruch 4, wobei der Ester des Peptids einen C-terminalen Ester umfasst.

7. Immunogenes Peptidfragment gemäß Anspruch 4, wobei das N-Acyl-Derivat des Peptids ein N-terminales Acyl-Derivat umfasst.

8. Nucleinsäuresequenz, die ein immunogenes Peptidfragment gemäß einem der Ansprüche 1 bis 3 codiert.

9. Nucleinsäuresequenz gemäß Anspruch 8, **dadurch gekennzeichnet, dass** sie die Nucleotidsequenz umfasst, die aus der Gruppe ausgewählt ist, die aus den Sequenzen SEQ ID Nr. 3, 5, 7 und 9 besteht.

10. Klonierungsvektor, der die Nucleinsäuresequenz von Anspruch 8 oder 9 umfasst.

11. Wirtszelle, **dadurch gekennzeichnet, dass** sie mit dem Klonierungsvektor gemäß Anspruch 10 transfiziert oder transformiert ist und vorzugsweise mit einem Klonierungsvektor, der die für ein MHC-Klasse-I-Allel codierende Nucleotidsequenz umfasst, cotransfiziert ist.

12. Wirtszelle gemäß Anspruch 11, **dadurch gekennzeichnet, dass** die Wirtszelle aus der Gruppe ausgewählt ist, die aus Mäuse-EL4- und -P8.15-Zellen und humanen BLM-Zellen besteht.

13. Wirtszelle gemäß Anspruch 11 oder 12, **dadurch gekennzeichnet, dass** sie eine antigenpräsentierende Zelle ist.

14. Wirtszelle gemäß einem der Ansprüche 11 bis 13, **dadurch gekennzeichnet, dass** sie auch costimulierende Moleküle erzeugt.

15. Impfstoff, **dadurch gekennzeichnet, dass** er ein immunogenes Peptid gemäß einem der Ansprüche 1 bis 7 oder ein Epitop davon umfasst.

16. Impfstoff gemäß Anspruch 15, **dadurch gekennzeichnet, dass** das Peptid mit einem pharmazeutisch annehmbaren Träger oder Verdünnungsmittel gemischt ist.

17. Impfstoff gemäß Anspruch 15 oder 16, **dadurch gekennzeichnet, dass** er eine antigenpräsentierende Zelle umfasst, die mit dem Peptid vorbeladen wurde.

18. Impfstoff, **dadurch gekennzeichnet, dass** er Wirtszellen gemäß einem der Ansprüche 12 bis 14 umfasst.

19. Impfstoff, **dadurch gekennzeichnet, dass** er eine Nucleinsäure gemäß Anspruch 8 oder 9 umfasst.

20. Impfstoff, **dadurch gekennzeichnet, dass** er den T-Zell-Rezeptor gegen die Peptide gemäß einem der Ansprüche 1 bis 7 oder Zellen, die diesen T-Zell-Rezeptor exprimieren, umfasst.

21. Impfstoff gemäß einem der Ansprüche 15 bis 20, **dadurch gekennzeichnet, dass** er auch eine oder mehrere Verbindungen umfasst, die aus der Gruppe ausgewählt sind, die aus einem Adjuvans, einem oder mehreren Cytokinen, Antikörpern, die gegen CD2, CD3, CD27, CD28 oder andere T-Zellen-

Oberflächenantigene gerichtet sind, und Helferepitopen zur Stimulation von CD4-positiven oder CD8-positiven T-Zellen besteht.

22. Verfahren zur Erzeugung von antigenreaktiven tumorinfiltrierenden Lymphocyten, **dadurch gekennzeichnet, dass** es die folgenden Schritte umfasst:

    a. Kultivieren von tumorinfiltrierenden Lymphocyten, die in einer Melanomprobe vorhanden sind;

    b. Isolieren der tumorinfiltrierenden Lymphocyten aus der Probe;

    c. Umsetzen der Lymphocyten mit einem immunogenen Peptid gemäß einem der Ansprüche 1 bis 7; und

    d. Isolieren der Lymphocyten, die an das Antigen binden.

23. Tumorinfiltrierender Lymphocyt, **dadurch gekennzeichnet, dass** er an ein immunogenes Peptid gemäß einem der Ansprüche 1 bis 7 binden kann.

24. Impfstoff, **dadurch gekennzeichnet, dass** er tumorinfiltrierende Lymphocyten gemäß Anspruch 23 umfasst.

25. Konjugat aus einem Peptid und einem nachweisbaren Marker, **dadurch gekennzeichnet, dass** das immunogene Peptid gemäß einem der Ansprüche 1 bis 7 verwendet wird.

26. Konjugat gemäß Anspruch 25, **dadurch gekennzeichnet, dass** der nachweisbare Marker ein Radionucleotid ist.

Revendications

1. Fragment peptidique immunogène de l'antigène associé au mélanome de SEQ ID n° 2, **caractérisé en ce que** ledit fragment comprend une séquence d'acides aminés choisie à partir du groupe composé de L-L-D-G-T-A-T-L-R-L, V-L-P-D-G-Q-V-I-W-V, T-W-G-Q-Y-W-Q-V, V-W-K-T-W-G-Q-Y-W-Q-V, K-T-W-G-Q-Y-W-Q-V-L, K-T-W-G-Q-Y-W-Q-V et T-W-G-Q-Y-W-Q-V-L, où ledit peptide immunogène est capable d'induire une lyse de cellules cibles par des lymphocytes infiltrant la tumeur.

2. Fragment peptidique immunogène selon la revendication 1, qui comprend une délétion, un remplacement, une inversion et/ou une addition.

3. Fragment peptidique immunogène selon la revendication 2, dans lequel ledit remplacement est choisi à partir du groupe comprenant des substitutions de Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn et Ile/Val.

4. Fragment peptidique immunogène selon une quelconque des revendications 1-3, dans lequel ledit peptide immunogène comprend un sel du peptide, un amide du peptide, un ester du peptide, un dérivé N-acyl du peptide ou un dérivé N-acétyl du peptide.

5. Fragment peptidique immunogène selon la revendication 4, dans lequel ledit amide du peptide comprend un amide C-terminal.

6. Fragment peptidique immunogène selon la revendication 4, dans lequel ledit ester du peptide comprend un ester C-terminal.

7. Fragment peptidique immunogène selon la revendication 4, dans lequel ledit dérivé N-acyl du peptide comprend un dérivé N-terminal acyl.

8. Séquence d'acide nucléique codant un fragment peptidique immunogène selon une quelconque des revendications 1-3.

9. Séquence d'acide nucléique selon la revendication 8, **caractérisée en ce qu'**elle comprend la séquence de nucléotides choisie à partir du groupe composé des séquences des SEQ ID n° 3, 5, 7 et 9.

10. Véhicule de clonage comprenant la séquence d'acide nucléique de la revendication 8 ou 9.

11. Cellule hôte, **caractérisée en ce qu'**elle est transfectée ou transformée avec le véhicule de clonage selon la revendication 10, de préférence cotransfectée avec un véhicule de clonage comprenant la séquence de nucléotides codant pour un allèle de MHC classe I.

12. Cellule hôte selon la revendication 11, **caractérisée en ce que** la cellule hôte est choisie à partir du groupe composé de cellules EL4 et P8-15 murines et de cellules BLM humaines.

13. Cellule hôte selon la revendication 11 ou 12, **caractérisée en ce qu'**elle est une cellule présentant un antigène.

14. Cellule hôte selon une quelconque des revendications 11-13, **caractérisée en ce qu'**elle produit également des molécules co-stimulantes.

15. Vaccin **caractérisé en ce qu'**il comprend un peptide immunogène selon une quelconque des revendications 1-7, ou un épitope de celui-ci.

16. Vaccin selon la revendication 15, **caractérisé en ce que** le peptide est mélangé avec des diluants ou des supports pharmaceutiquement acceptables.

17. Vaccin selon la revendication 15 ou 16, **caractérisé en ce qu'**il comprend une cellule présentant un antigène, qui a été préchargé avec le peptide.

18. Vaccin, **caractérisé en ce qu'**il comprend des cellules hôtes selon une quelconque des revendications 12-14.

19. Vaccin, **caractérisé en ce qu'**il comprend un acide nucléique selon la revendication 8 ou 9.

20. Vaccin, **caractérisé en ce qu'**il comprend le récepteur des cellules T contre les peptides selon une quelconque des revendications 1-7, ou des cellules exprimant ledit récepteur des cellules T.

21. Vaccin selon une quelconque des revendications 15-20, **caractérisé en ce qu'**il comprend également un ou plusieurs composés choisis à partir du groupe comprenant un adjuvant, une ou plusieurs cytokines, des anticorps dirigés contre des antigènes de surface des cellules T CD2, CD3, CD27, CD28 ou autres et des épitopes auxiliaires pour stimuler les cellules T CD4+ ou CD8+.

22. Procédé pour engendrer des lymphocytes infiltrant une tumeur réactifs pour l'antigène, **caractérisé en ce qu'**il comprend les étapes de :

    a. mettre en culture des lymphocytes infiltrant la tumeur présents dans un échantillon de mélanome ;
    b. isoler les lymphocytes infiltrant la tumeur à partir de l'échantillon ;
    c. faire réagir lesdits lymphocytes avec un peptide immunogène selon une quelconque des revendications 1-7 ; et
    d. isoler les lymphocytes se liant audit antigène.

23. Lymphocyte infiltrant une tumeur, **caractérisé en ce qu'**il est capable de se lier à un peptide immunogène selon une quelconque des revendications 1-7.

24. Vaccin, **caractérisé en ce qu'**il comprend des lymphocytes infiltrant une tumeur selon la revendication 23.

25. Conjugué d'un peptide et d'un marqueur détectable, **caractérisé en ce que** le peptide immunogène selon une quelconque des revendications 1-7 est utilisé.

26. Conjugué selon la revendication 25, **caractérisé en ce que** le marqueur détectable est un radionucléotide.

Figure 1

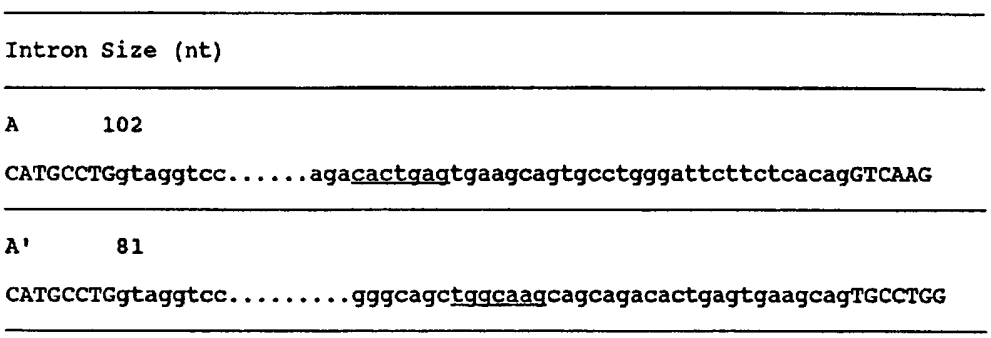
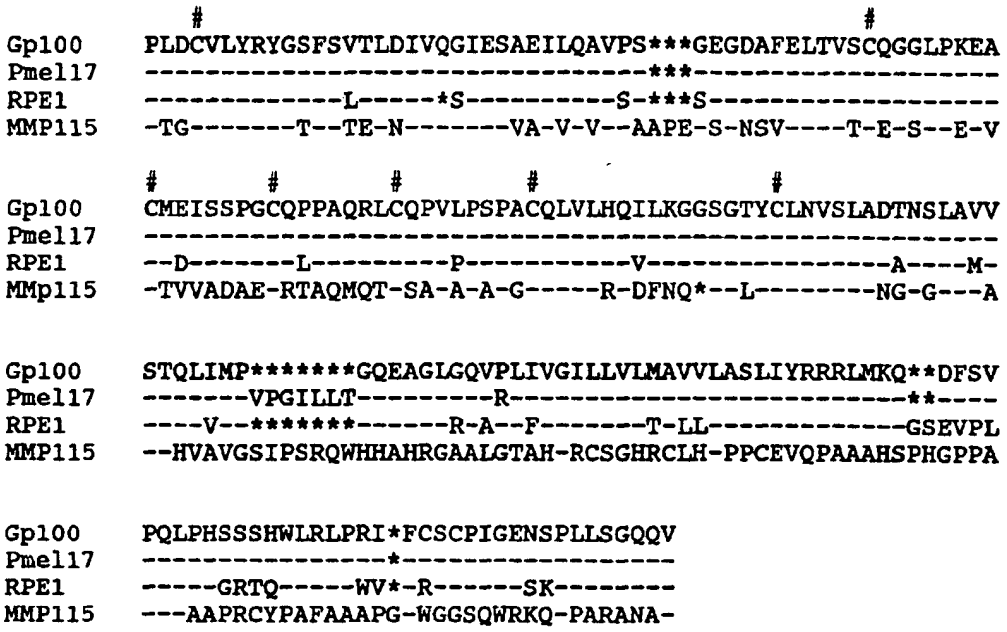
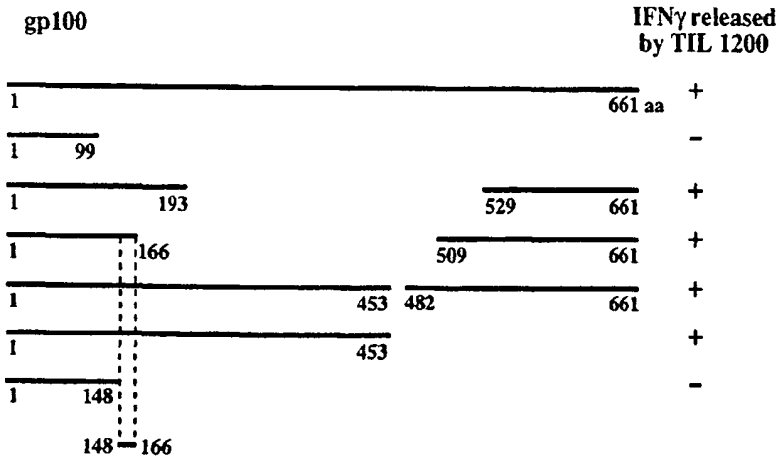

Intron Size (nt)
A 102
CATGCCTGgtaggtcc.....agacactgagtgaagcagtgcctgggattcttctcacagGTCAAG
A' 81
CATGCCTGgtaggtcc.....gggcagctggcaagcagcagacactgagtgaagcagTGCCTGG

Figure 2


Gp100 PLDCVLYRYGSFSVTLDIVQGIESAEILQAVPS***GEGDAFELTVSCQGGLPKEA
Pmel17 ----------***----------
RPE1 ----------L-----*S----------S-***S----------
MMP115 -TG-------T--TE-N-------VA-V-V--AAPE-S-NSV----T-E-S--E-V
Gp100 CMEISSPGCQPPAQRLCQPVLPSPACQLVLHQILKGGSGTYCLNVSLADTNSLAVV
Pmel17 ----------
RPE1 --D-------L---------P----------V----------A----M-
MMp115 -TVVADAE-RTAQMQT-SA-A-A-G-----R-DFNQ*--L--------NG-G---A
Gp100 STQLIMP*******GQEAGLGQVPLIVGILLVLMAVVLASLIYRRRLMKQ**DFSV
Pmel17 -------VPGILLT---------R----------**----
RPE1 ----V--*******------R-A--F-------T-LL----------GSEVPL
MMP115 --HVAVGSIPSRQWHHAHRGAALGTAH-RCSGHRCLH-PPCEVQPAAAHSPHGPPA
Gp100 PQLPHSSSHWLRLPRI*FCSCPIGENSPLLSGQQV
Pmel17 ----------*----------
RPE1 -----GRTQ-----WV*-R------SK--------
MMP115 ---AAPRCYPAFAAAPG-WGGSQWRKQ-PARANA-

A


gp100
IFNγ released by TIL 1200
1
661 aa
+
1
99
-
1
193
529
661
+
1
166
509
661
+
1
453
482
661
+
1
453
+
1
148
-
148
166

B

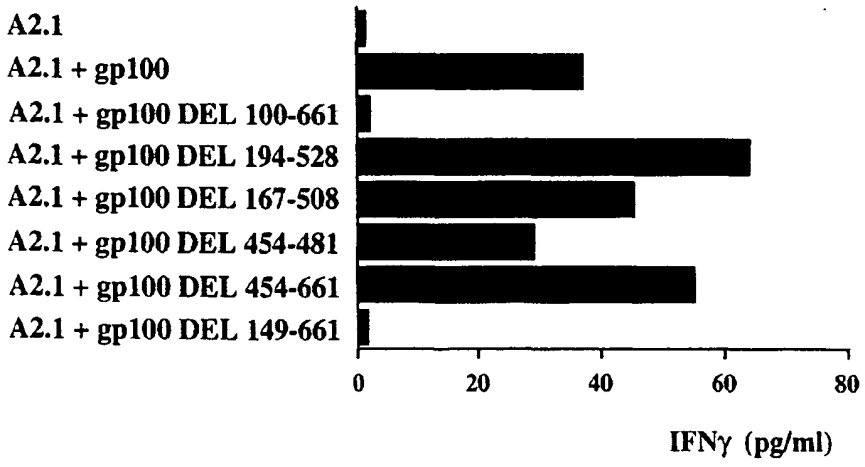
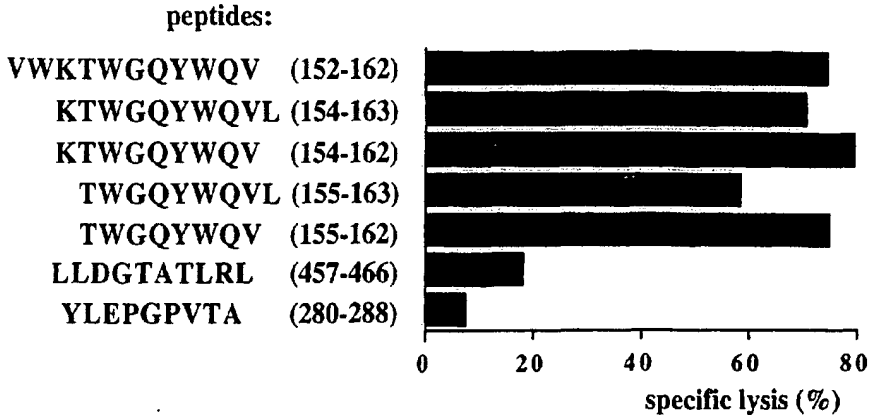
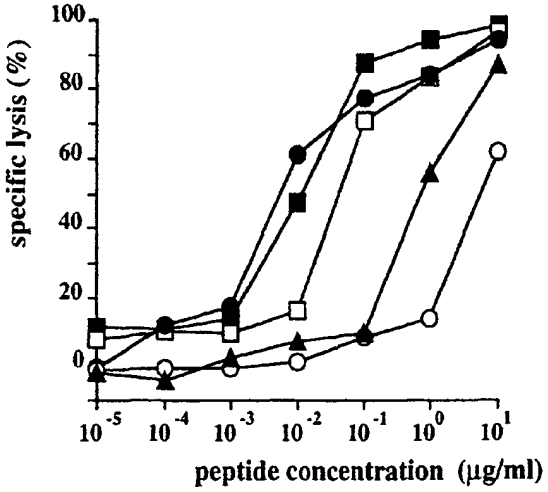

A2.1
A2.1 + gp100
A2.1 + gp100 DEL 100-661
A2.1 + gp100 DEL 194-528
A2.1 + gp100 DEL 167-508
A2.1 + gp100 DEL 454-481
A2.1 + gp100 DEL 454-661
A2.1 + gp100 DEL 149-661
0
20
40
60
80
IFNγ (pg/ml)

figure 3

A


peptides:
VWKTWGQYWQV (152-162)
KTWGQYWQVL (154-163)
KTWGQYWQV (154-162)
TWGQYWQVL (155-163)
TWGQYWQV (155-162)
LLDGTATLRL (457-466)
YLEPGPVTA (280-288)
0
20
40
60
80
specific lysis (%)

B


specific lysis (%)
100
80
60
40
20
0
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
peptide concentration (µg/ml)

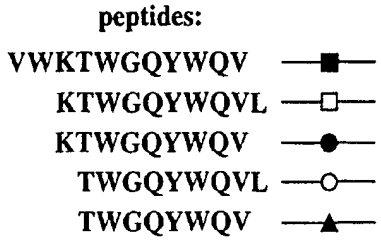

peptides:
VWKTWGQYWQV
KTWGQYWQVL
KTWGQYWQV
TWGQYWQVL
TWGQYWQV

figure 4

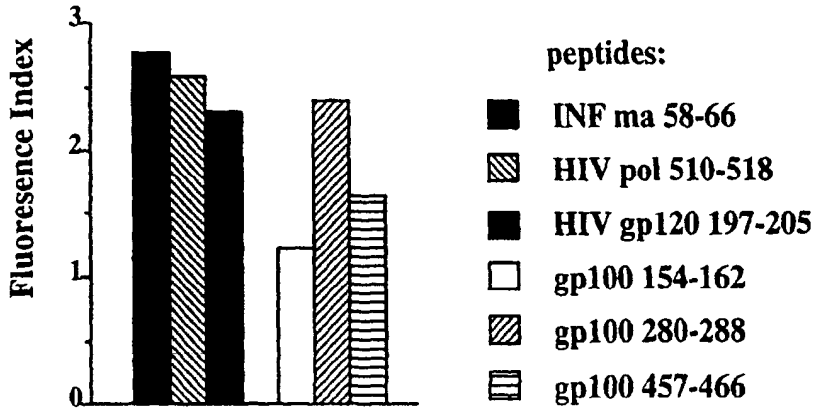

Fluoresence Index
3
2
1
0
peptides:
INF ma 58-66
HIV pol 510-518
HIV gp120 197-205
gp100 154-162
gp100 280-288
gp100 457-466

figure 5